ELECTROCHEMICAL ANALYSIS OF COENZYME Q_{10} AND REDUCED COENZYME Q_{10}

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This application is based on and claims priority from U.S. Provisional Patent Application Serial No. 60/273,684, Peter H. Tang, Ton deGrauw, Michael V. Miles, filed March 6, 2001.

Field of Invention

[0001] The present invention relates to a method of electrochemical analysis of an aqueous solution containing materials having quinone and hydroquinone moieties. More particularly, the present invention relates to an electrochemical apparatus and method for the simultaneous measurements of coenzyme Q_{10} and the reduced form of coenzyme Q_{10} concentrations in human plasma samples.

Background

[0002] Coenzyme Q_{10} (CoQ_{10}), commonly known as ubiquinone-10, is present in blood and all body tissues in both reduced and oxidized forms. CoQ_{10} has an important function in the mitochondrial electron transport chain (ETC) as an electron acceptor and as an electron donor. It is believed that the ETC is comprised of five multi-subunit enzyme complexes, in which CoQ_{10} and cytochrome c act as shuttles between complex I and III and II and III. Complex I (NADH-ubiquinone oxidoreductase) transfers electron from NADH or NADPH to CoQ_{10}. Complex II (succinate-ubiquinone oxidoreductase) transfers electron from FADH or FMNH to CoQ_{10}. Complex III (ubiquinol-ferrocytochrome c oxidoreductase) transfers electron from reduced CoQ_{10} ($CoQ_{10}H_2$) to cytochrome c. In addition, $CoQ_{10}H_2$ is one of the

antioxidants that protect cells against lipid peroxidation. In the circulation, CoQ_{10} is mainly carried by lipoproteins, where it is predominantly present in the reduced form. The $CoQ_{10}H_2$ in low density lipoprotein (LDL) is, however, easily oxidized to CoQ_{10}. In fact, $CoQ_{10}H_2$ is the first antioxidant to be depleted when LDL is subjected to oxidative stress *in vivo*. It has been postulated that $CoQ_{10}H_2$ prevents the initiation and/or the propagation of lipid peroxidation in plasma lipoproteins and biological membranes. The antioxidative activity of $CoQ_{10}H_2$ depends not only on its concentration but also on its redox status. Recent reports have suggested the percentage of $CoQ_{10}H_2$ in total plasma concentration of CoQ_{10} (TQ_{10}) may be lower in patients with atherosclerosis, hyperlipidemia and coronary artery disease, and may be a useful biomarker of oxidative stress. Thus, the measurement of $CoQ_{10}H_2$ and CoQ_{10} is of primary importance for clinical diagnosis.

[0003] Previous $CoQ_{10}H_2$ studies have encountered the problem of $CoQ_{10}H_2$ stability during sample handling, storage, and processing [1, 2, 3, 4, 5, 6, 7]. These studies indicate that $CoQ_{10}H_2$ is unstable in blood, plasma, and hexane extracts at room temperature. Subsequently, the $CoQ_{10}H_2$:TQ_{10} ratio changes considerably within an hour after the blood sample has been obtained. The lability of $CoQ_{10}H_2$ is due to the hydroquinone moiety which is sensitive to oxygen, and at room temperature, it spontaneously oxidizes to CoQ_{10} at a rate of ~2 nM per min. This problem is very obvious in many studies which have reported wide variability in the $CoQ_{10}H_2$:TQ_{10} ratio in biological fluids [1, 2, 3, 4, 5, 6, 7]. It is believed that sample preparation has a profound effect on the redox status of CoQ_{10} and that utmost care is required to ensure reliable estimates of the $CoQ_{10}H_2$:TQ_{10} ratio. Recently, investigators have recommended that each plasma sample be thawed individually, extracted, and

analyzed as a continuous process to minimize $CoQ_{10}H_2$ oxidation [8, 9, 10]. This is obviously very impractical for analyzing significant numbers of clinical specimens.

[0004] Several in-line post-column reduction methods of CoQ_{10} to $CoQ_{10}H_2$ have also been reported for simultaneous measurements by electrochemical detection [3, 4]. The complex instrumentation and techniques used in those reports limit their practical value.

[0005] In earlier studies, biological fluid samples were converted into either CoQ_{10} using an oxidizing reagent such as hydrogen peroxide or ferric chloride, or converted into $CoQ_{10}H_2$ using a reducing agent such as sodium tetrahydroborate or sodium dithionite (Table 1). In practice, however, since $CoQ_{10}H_2$ is easily oxidized when exposed to air, $CoQ_{10}H_2$ is susceptible to pre-analytical degradation and analytical error.

[0006] Therefore, we developed a simple and rapid procedure using an isocratic HPLC-EC method for simultaneous determination of CoQ_{10} and $CoQ_{10}H_2$ in human samples. An electrochemical (EC) detector is preferred for detection of $CoQ_{10}H_2$ due to its high sensitivity. The electrochemical reactions are monitored at electrodes that measure the current produced by the reduction of the quinone group of CoQ_{10} or by the oxidation of the hydroquinone group of $CoQ_{10}H_2$ (Figure 1). This method may be used to investigate conditions by which the $CoQ_{10}H_2$:TQ_{10} ratio can be reliably measured.

Summary of the Invention

[0007] The present invention relates to an electrochemical detection apparatus and method for electrochemically determining the concentration of quinones and

hydroquinones, such as CoQ_{10} and $CoQ_{10}H_2$, in biological fluids. The detection method is sufficiently sensitive to permit the fluid sample to be as small as 100 μL.

[0008] The apparatus comprises a coulometric guard cell, which operates in an oxidative mode at about +700 mV or higher. This is arranged in series with an analytical cell that is placed after the guard cell. The analytical cell consists essentially of a series of at least two coulometric electrodes. The first electrode operates in a reductive mode at about –650 mV or lower. The second electrode operates in an oxidative mode at a potential that simultaneously detects and coulometrically measures electrochemically reversible materials in the sample solution. The operating potential for this second electrode is generally about +500 mV or higher. The guard cell and analytical cell are arranged so as to define collectively at least one flow channel for the sample solution to pass through. For many applications, a liquid chromatographic column is placed before the coulometric guard cell in order to achieve time-spaced separation of materials.

[0009] The sample itself comprises a mixture of electrochemically reversible materials, such as quinones and hydroquinones, contained in an aqueous solution. The aqueous solutions include, but are not limited to, water, juices, wine, milk, and aqueous pharmaceutical aqueous formulations. More particularly, the sample solution comprises a mixture of CoQ_{10} and $CoQ_{10}H_2$ in heparinized human plasma which is diluted with 1-propanol. Although the present invention is primarily applicable to testing biological fluids such as plasma, serum, urine, CSF, breast milk, amniotic fluid, and blood, it may also be used to analyze solid matrices such as tissues, cell lysate and solid pharmaceutical formulations.

[0010] The method for simultaneous analysis a mixture of electrochemically reversible materials comprises dissolving the materials in a solution of 1-propanol.

Once dissolved, the materials are passed through a liquid chromatographic column for achieving time-spaced separation of the materials eluted from the column. As these materials are eluted off the column, they are then oxidized as they pass through the coulometric guard cell. Once through the coulometric guard cell, the materials pass through an analytical cell consisting essentially of a series of at least two coulometric electrodes, wherein the first electrode operates in a reductive mode and the second electrode operates in an oxidative mode at a potential so as to detect and coulometrically measure electrochemically reversible materials in said sample. These coulometric cells are arranged in series to define collectively at least one flow channel for the sample solution.

[0011] An in-line pre-column reduction cell may also be placed between the injection port and the analytical column. This permits transformation of CoQ_{10} into $CoQ_{10}H_2$, and vice versa. The yield of electrochemical reduction is approximately 99%.

Description of Drawings

[0012] Figure 1 shows the electrochemical reactivity of $CoQ_{10}H_2$ and CoQ_{10}.

[0013] Figure 2 is a schematic diagram of the HPLC-EC system. This system can be operated in three different modes as described in the present invention.

[0014] Figure 3A is a chromatogram showing two oxidation peaks for standards of CoQ_{10} and CoQ_9 at ~5.6 and ~6.9 min, respectively.

[0015] Figure 3B is a chromatogram showing two oxidation peaks for standards of CoQ_9H_2 and $CoQ_{10}H_2$ at ~3.6 and ~4.0 min, respectively. Pre-column reduction mode was operated to transform CoQ_{10} and CoQ_9 to $CoQ_{10}H_2$ and CoQ_9H_2, respectively.

[0016] Figure 4 shows plots of calibration curves for CoQ_{10} and $CoQ_{10}H_2$. Standards of CoQ_{10} were dissolved in 1-propanol.

[0017] Figure 5 is a graph comparing the effects of anticoagulant heparin and EDTA on $CoQ_{10}H_2$ stability among 13 blood samples collected in paired Vacutainer®s containing heparin and EDTA.

[0018] Figure 6 is a graph comparing the effect of heparin and EDTA on $CoQ_{10}H_2$ in blood specimens over 6 hours. Blood specimens from 5 healthy volunteers were collected in paired VACUTAINERS containing heparin or EDTA and stored under refrigerated conditions.

[0019] Figure 7 is a stability profile of $CoQ_{10}H_2$ in blood specimens over a period of 72 hours.

[0020] Figure 8 is a graph showing the stability of $CoQ_{10}H_2$ in 1-propanol extract at 0-4°C.

[0021] Figure 9 shows a chromatograms of a patient's plasma extracts with and without CoQ_9 internal standard.

[0022] Figure 10A shows a chromatogram of a patient's plasma extract before CoQ_{10} supplementation.

[0023] Figure 10B shows a chromatogram of a patient's plasma extract after CoQ_{10} supplementation at a dosage of 60 mg/day.

Detailed Description of the Invention

[0024] This invention relates to an apparatus for electrochemically analyzing an aqueous sample solution comprising electrochemically reversible materials in solution. In its broadest sense, electrochemically reversible materials are those which can be oxidized and/or reduced by the transfer of electrons. There may also be the

transfer of protons (H^+) as well in order to form a chemically neutral species as the final product of the electron transfer reaction. Generally, the apparatus comprises an HPLC instrument, which includes a solvent delivery module (pump), an injector, in-line filters, guard column and an analytical HPLC column for separation of the materials. In addition, immediately preceding the analytical column is at least one coulometric guard cell that can be used to oxidize or reduce the electrochemically active species. Following the analytical column is a series of in-line electrochemical cells for reduction and/or oxidation as well as analysis of the electrochemically active species. The coulometric guard cell preceding the analytical column and the series of post-column in-line electrochemical cells define collectively at least one flow channel for the sample solution.

[0025] The following methods are used to prepare the necessary standards:

Materials

[0026] CoQ_9 and CoQ_{10} are obtained from Sigma, St. Louis, Missouri. HPLC grade methanol, ethanol, 1-propanol, 2-propanol and hexane are obtained from Fisher, and used without further purification. Sodium acetate and analytical grade acetic acid are also obtained from Fisher.

Preparation of Standard Solutions

[0027] The preparation work is carried out under a dim light to avoid photochemical decomposition of CoQ_{10} and CoQ_9. To prepare a 5 ug/ml of a CoQ_{10} working solution, 10 mg of CoQ_{10} are dissolved in 10 mL of hexane and diluted to 100 mL with 1-propanol. The solution is thoroughly vortexed until dissolution is complete. A working solution is then prepared by diluting with 1-propanol to 5 ug/mL. The

concentration of the working solution is then calculated by reading the absorbance at the spectrophotometer (275 nm, 1 cm light path quartz cuvette using epsilon = 14,200). A series of calibration and control solutions is then prepared with the appropriate volume of 1-propanol to have final concentrations of 10, 100, 500, 1000, 2000, and 4000 ng/mL for CoQ_{10} calibration solution, and the control solutions have final concentrations of 75, 750, and 1500 ng/mL CoQ_{10}. The CoQ_9 is chosen as an internal standard. To prepare a CoQ_9 solution, 2 mg of CoQ_9 are dissolved in 100 mL of 1-propanol. The CoQ_9 solution is thoroughly vortexed until dissolution is complete. A working solution of CoQ_9 is then prepared by diluting with 1-propanol to a concentration of 2 ug/mL. All the solutions are stored in 1.8 ml polypropylene tubes (Sarstedt, Newton, NC, USA) at -20°C and used throughout the study.

HPLC-EC System

[0028] The HPLC-EC system is depicted in Figure 2. For chromatography with coulometric analyses, the HPLC system consists of an ESA Model 582 Solvent Delivery Module (Bedford, MA) equipped with a double plunger reciprocating pump, an AS3000 variable-loop autosampler (Thermo Separation Products, Freemont, CA, USA), an analytical column, an ESA CouloChem II Model 5200A electrochemical detector, and a Dell Pentium II 35OMz computer/controller with ChromQuest software (Thermo Separation Products). The system also comprises two coulometric guard cells (E1 and E2, pre- and post-column, with respect to the analytical column, see Figure 2) and an analytical cell, E3 and E4. There are also two in-line filters, wherein one in-line filter is placed before the guard cell E1 and the second in-line filter is placed between the analytical column and the guard cell (E2).

[0029] The analytical column used is a reverse-phase Microsorb-MV column (5 μm, 4.6 mm x 15 cm). A reverse phase C 18 guard column (5-μm, 10 x 4.6 mm) is used to protect the analytical column. The analytical column temperature is at room temperature. The AS3000 injector is set at needle height of 1.5 mm and injection volume of 20 μl for each sample. The cooler temperature of the autosampler is at 0-4°C.

[0030] Each guard cell (E1 and E2) is a single coulometric cell (ESA Model 5020). The guard cells are installed before and after the analytical column (Figure 2). The analytical cell (E3 and E4, ESA Model 5010) is connected in series to the post-column guard cell E2. The analytical cell consists of a series of two coulometric electrodes. The first electrode is operated in the reduction mode (about -650 mV or lower) for reduction of CoQ_{10} and he second electrode is operated in the oxidation mode (about +500 mV or higher) for detection of $CoQ_{10}H_2$.

[0031] The mobile phase for the isocratic elution of CoQ_{10} is prepared as follows: sodium acetate trihydrate (6.8 g), 15 mL of glacial acetic acid and 15 mL of 2-propanol are added to 695 ml of methanol and 275 ml of hexane. Mobile phase is filtered by 0.2 μm pore-sized, 47mm nylon filter or analogous filter. Mobile phase has a pH value of 6. The flow rate is 1.0 mL/min.

Operation of Coulometric System

[0032] This system can be operated at three different modes for three different purposes: 1) precolumn reduction mode for measuring total $CoQ_{10}H_2$; 2) precolumn oxidation mode for measuring total CoQ_{10}; and 3) precolumn off mode for simultaneous determination of $CoQ_{10}H_2$ and CoQ_{10}.

[0033] In order to determine the optimal applied-voltage for EC detection, a hydrodynamic voltammogram is obtained by analyzing a solution of CoQ_{10} (4000 ng/mL) at different voltage settings. Anodic currents and cathodic currents reach maximum responses at applied voltages of +500 mV and -600 mV, respectively. Hence, the detection potential is maintained at +500 mV or higher vs. the hydrogen/palladium reference electrode.

[0034] For measuring total $CoQ_{10}H_2$, the precolumn guard cell is operated in the reduction mode (-800 mV or lower) in order to transform CoQ_{10} to $CoQ_{10}H_2$. The reduction mode is also used to establish a calibration curve of $CoQ_{10}H_2$.

[0035] For measuring total CoQ_{10}, the precolumn guard cell is operated in the oxidation mode (+700 mV or higher) in order to oxidize $CoQ_{10}H_2$ to CoQ_{10}.

[0036] For measuring $CoQ_{10}H_2$ and CoQ_{10} simultaneously, the precolumn guard cell is operated in the off mode (no current flows into the guard cell). The postcolumn guard cell is operated at oxidation mode (+700 mV or higher) to oxidize any electrochemically active elutes.

Sample Handling and Processing

Use of Heparin as an Anticoagulant for Blood Collection

[0037] According to the present invention, venous blood is collected into a VACUTAINER containing heparin as anticoagulant and mixed gently by inversion 5-6 times. At this point the blood-heparin tube is securely capped, and is then placed in ice and/or kept in refrigeration prior to processing. The blood-heparin tube should be processed within 4 hours. Blood samples should not be collected in tubes containing metal chelators, such as ethylenediaminetetraacetic acid (EDTA). The blood-heparin tube is subject to centrifugation at an appropriate speed such as 3000 rpm for 10

minutes at 4°C. Heparinized plasma is separated from red blood cells and placed in a capped polypropylene tube and immediately stored at -75°C or below until analysis.

Extraction of $CoQ_{10}H_2$ and CoQ_{10}

[0038] Each frozen sample is thawed at room temperature, and 100 µl of this sample is placed in a 1.5-ml capped polypropylene tube containing 50 µl of internal standard solution (CoQ_9, 2µg/mL in 1-propanol). All the tubes are kept in an ice-bath. This is mixed with 850 µl of cold 1-propanol. The tubes are vortexed for 2 minutes on a mechanical vortexer and centrifuged for 10 min at 0-4°C. The resulting supernatant is separated from the precipitate and transferred to an autosampler glass vial. The sample vial is immediately placed in the autosampler tray at 0-4°C. A batch of up to 20 samples can be extracted and processed at the same time. An aliquot of 20 µl of 1-propanol extract from a vial is injected immediately onto an automated HPLC. Twenty samples can be analyzed sequentially, within 4 hours. If an error has occurred in the system, these sample vials can be resealed and immediately restored at -75°C or below for further investigation.

Analysis of CoQ_{10} and $CoQ_{10}H_2$

Quantitation of CoQ_{10} and $CoQ_{10}H_2$

[0039] Standard solutions of CoQ_{10} are prepared as described above. Coulometric analysis and subsequent measurements of current responses are performed as described above. Two oxidation peaks are observed for CoQ_9 and CoQ_{10} at approximately 5.6 min. and 6.9 min, respectively (Figure 3). Peak height measurements for each calibration curve are obtained by using the ChromQuest software. The peak height ratios of CoQ_{10}/ CoQ_9 are used to obtain least squares

linear regression equations, which are used to calculate the CoQ_{10} concentrations of the frozen control samples and patient samples. A linear response up to a concentration of 4 μg/mL of CoQ_{10} is obtained when the peak height ratios are plotted versus CoQ_{10} concentration (Figure 4).

[0040] The same solutions of CoQ_{10} used in the section describing Quantitation of CoQ_{10} are reduced electrochemically for $CoQ_{10}H_2$ measurements as described above. Coulometric analysis and subsequent measurements of current responses are performed as described above. Two reduction peaks are observed for CoQ_9H_2 and $CoQ_{10}H_2$ at approximately 3.6 and 4.1 min, respectively (Figure 3). The peak height measurements for each calibration are obtained by using the ChromQuest software. Peak height measurements of CoQ_9 obtained in the section of "Quantitation of CoQ_{10}" are used herein. The peak height ratios of $CoQ_{10}H_2/CoQ_9$ are used to obtain a least squares linear regression equation, which is then used to calculate the $CoQ_{10}H_2$ concentrations of the frozen control samples and patient samples. A linear response up to a concentration of 4μg/mL of $CoQ_{10}H_2$ is obtained when the peak height ratios are plotted versus $CoQ_{10}H_2$ concentration (Figure 4).

Heparin versus EDTA

[0041] For $CoQ_{10}H_2$ analysis, many previous studies have wrongly used plasma samples anticoagulated with EDTA. The choice of anticoagulant has an effect on oxidation of $CoQ_{10}H_2$ (Figure 5). Each patient's blood specimen is drawn into two paired VACUTAINERS containing heparin or EDTA. Although the time of sample handling and delivery may be varied from patient to patient, each pair of blood-containing tubes are handled and processed identically. The mean values of $CoQ_{10}H_2$ and TQ_{10} from the heparinized plasma samples were 748(±346) and 770(±353)

ng/mL, respectively, but somewhat poorer for those in EDTA-plasma with the mean values of 643(±321) and 696(±326) ng/mL, respectively. For the ratio of $CoQ_{10}H_2$:TQ_{10} the mean values of heparinized plasma, 97.0(±1.4)%, were better than those in EDTA-plasma, 91.5(±4.4)%. These results clearly indicate that ratios and levels of $CoQ_{10}H_2$ and TQ_{10} in heparinized plasma are consistently higher than in plasma anticoagulated with EDTA ($p<0.001$ for difference in paired samples).

[0042] When blood samples in vacutainers are opened and kept refrigerated, the $CoQ_{10}H_2$:TQ_{10} ratios (n=30) in EDTA samples decreased by ~30% over 7h, whereas the $CoQ_{10}H_2$:TQ_{10} ratios in heparinized samples were stable over the same period with variation of less than 3% (median 0.949; range 0.927-0.970; n=30). There was as significant difference of $CoQ_{10}H_2$:TQ_{10} ratios in heparin in EDTA (P=0.028). The observed findings therefore reflect the effect of air-exposure on EDTA samples during the blood sampling procedure. The results also indicate that delays in processing and analyzing EDTA samples would result in lowered and variable $CoQ_{10}H_2$:TQ_{10} ratios.

[0043] Using the presently described procedure, the $CoQ_{10}H_2$:TQ_{10} ratio was >95% in the plasma specimens of normal volunteers. Heparin seems to possess antioxidant properties, but a previous study has shown that heparin has no direct antioxidant properties even at concentrations far higher than those usually used therapeutically. Thus, other mechanisms not strictly antioxidant-type may be involved in heparin-mediated protection on $CoQ_{10}H_2$. While not being bound by theory, it is thought that either heparin is interacting with a lipoprotein to enhance antioxidant protection perhaps through release of superoxide dismutase, or the chelation of metal ions by EDTA is limiting the activity of antioxidant metalloenzymes.

Temperature control

[0044] $CoQ_{10}H_2$ is not stable and is oxidized in air at room temperature. $CoQ_{10}H_2$ is unstable in whole blood, plasma and n-propanol extract when EDTA is used as anticoagulant. EDTA-anticoagulated blood loses approximately 15% of $CoQ_{10}H_2$ within 4 hours when stored in refrigeration (4°C), whereas only <1% loss of $CoQ_{10}H_2$ occurs in heparin-anticoagulated blood under the same conditions (Figure 6). When fresh blood samples in closed heparin vacutainers are kept refrigerated, no significant difference in $CoQ_{10}H_2$:TQ_{10} ratios was observed between samples stored for 0 h and 24 h (P=0.052). The median $CoQ_{10}H_2$:TQ_{10} ratio stored for 24 h was 0.955 (range 0.923-0.972; n=8) in comparison to the initial value of 0.964 (range 0.935-0.978; n=8). The $CoQ_{10}H_2$:TQ_{10} ratios decreased by ~6% after 48 h and by ~28% after 72 h. Storage of blood specimens in VACUTAINERS at +4°C (in refrigeration) prior to separation of plasma from blood cells can extend the stability of $CoQ_{10}H_2$ to 24 hours without a significant loss (<3%, See Figure 7). Since blood $CoQ_{10}H_2$ in closed heparin VACUTAINERS kept refrigerated is stable for at least 24 hours, an overnight shipment of a blood sample on ice pack is acceptable for testing. When plasma is kept frozen at -75°C or below, $CoQ_{10}H_2$ is stable for at least 6 months and the $CoQ_{10}H_2$:TQ_{10} ratio does not change significantly during this period. Since measurements of $CoQ_{10}H_2$ from frozen samples is unfeasible, frozen samples must be thawed, extracted, and analyzed quickly to ensure minimal $CoQ_{10}H_2$ oxidation during the measurement process. Okamoto *et al* [10] reported the percentage of $CoQ_{10}H_2$ with respect to the TQ_{10} was constant in plasma for one day when kept at 2°C and -10°C. When extracted from plasma and prepared in ethanol solution, however, any $CoQ_{10}H_2$ in solution rapidly oxidized.

[0045] An optimized HPLC system for measuring $CoQ_{10}H_2$ sequentially within 4 hours shows that 3.75% of $CoQ_{10}H_2$ in 1-propanol extract oxidizes in an hour (at a rate of ~2 nM/min) at room temperature, whereas, the loss of $CoQ_{10}H_2$ is only 1% in 5 hours when the temperature is set at 0-4°C (Figure 8).

Extraction with 1-Propanol

[0046] Using 1-propanol as an extracting solvent allows fast and simple sample processing. The procedure has been simplified to a single extraction and no evaporation process is needed. This allows for efficient sample handling. Extraction of $CoQ_{10}H_2$ and CoQ_{10} from a variety of biological matrices is made facile with 1-propanol, because it can be mixed with water in any proportion. Additionally, $CoQ_{10}H_2$ and CoQ_{10} have highest solubility in 1-propanol relative to other alcohols. Thus, 1-propanol is an effective extraction solvent (Table 2). The 1-propanol extract can be directly analyzed by the described HPLC-EC method. Furthermore, this procedure takes advantage of the use of CoQ_9 as an internal standard. Because the levels of CoQ_9 in human blood are negligible, it is desirable to spike control samples with this CoQ_9 to validate the extraction process.

High Specificity

[0047] Using a series of postcolumn guard cells and analytical cells operating within the stated oxidation and reduction potentials provides a high degree specificity to the detector system, and only those compounds capable of undergoing a reversible reduction-oxidation at the low potentials used are capable of being detected by the last electrode. Accordingly, as a result of a compromise involving the mobile phase composition, flow-rate and concentration of the supporting electrolyte, an excellent

separation of an authentic mixture of CoQ_9, CoQ_{10} and $CoQ_{10}H_2$ is obtained on a Microsorb-MV C18 column (15 cm x 4.6 mm) using a mobile phase (pH ~6) containing 0.05M sodium acetate at a flow-rate of 1.0 ml/min. A patient's sample was obtained according to the current method, wherein the retention times were 4.1 min for $CoQ_{10}H_2$, 5.6 min for CoQ_9 and 6.9 min for CoQ_{10} (Figure 9). The procedure described herein is useful for investigating the effect of CoQ_{10} supplementation on patients with metabolic disorders (Figure 10).

Reliable measurement

[0048] $CoQ_{10}H_2$ is generally obtained by chemical reduction of the commercially available CoQ_{10}. This requires a strong reducing agent such as sodium tetrahydroborate. The reduced form is unstable at room temperature and quickly becomes oxidized, so a fresh solution of $CoQ_{10}H_2$ must prepared daily. All these procedures are time consuming, labor-intensive, tedious and unsuitable for routine determinations. An alternative procedure using electrochemical reduction to obtain the reduced form relies on a coulometric detector which is able to provide more than 99% yield of the desired electrochemical reaction (Table 3) and thus providing a calibration curve for measuring the $CoQ_{10}H_2$ concentration.

[0049] The calibration curves for reduced CoQ_{10} and oxidized CoQ_{10} were linear (r = 0.999) over the concentration range 10-4000 ng/ml. The detection limits of reduced CoQ_{10} and oxidized CoQ_{10} are about 5 ng/mL (S/N= 3) (Figure 4). This simplified 1-propanol extraction procedure is reproducible, allows for a recovery of CoQ_{10}, and allows for the assessment of the $CoQ_{10}H_2$ content from a standard concentration curve. Quantitative recovery of CoQ_{10} (100%) using 1-propanol solvent has been

obtained. The inter-day and intra-day assay variations (CVs) are less than 10% over a range 10-4,000 ng/mL (Table 4).

Time Requirement

[0050] The chromatography run time for each specimen is typically less than 10 minutes (Figure 9). The total time required for a complete sample analysis is less than 30 minutes. This method allows at least 40 samples to be analyzed in an 8 hour working day, and 24 samples could be completed for overnight analysis.

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